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Award Number: DAMD17-02-1-0309

TITLE: Characterization of Receptors for Peptides Homing to the  
Vasculature of the Breast Carcinomas by Display Cloning

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REPORT DATE: June 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20031117 004

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> June 2003	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (15 May 2002 - 15 May 2003)	
<b>4. TITLE AND SUBTITLE</b> Characterization of Receptors for Peptides Homing to the Vasculature of the Breast Carcinomas by Display Cloning			<b>5. FUNDING NUMBERS</b> DAMD17-02-1-0309	
<b>6. AUTHOR(S)</b> Lianglin Zhang, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> The Burnham Institute La Jolla, California 92037  E-Mail: lzhang@burnham.org			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b> Original contains color plates: All DTIC reproductions will be in black and white.				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b> Using in vivo screening of phage-displayed peptide libraries, a pentapeptide, CREKA, which homes to vasculature of breast tumor in mice was identified. The CREKA-displaying phage homes to breast tumors in mice with a 100-fold selectivity over non-recombinant phage. The homing of the CREKA phage was inhibited by its cognate synthetic peptide. The CREKA-displaying phage and fluorescein-labeled CREKA peptide were taken up by tumor tissue, but not by control tissues, and they co-localized with septa of connective tissue in tumors. Using the CREKA peptide in expression cloning with a phage-displayed cDNA library yielded a phage that specifically bound to the peptide. The cDNA coded for a fragment of the collagen IV alpha 2 chain with typical Gly-X-Y repeats. CREKA phage bound avidly to immobilized collagen. Heat denaturation of the triple helical structure of collagen enhanced CREKA binding to collagen. These results indicate that the CREKA peptide homes to tumors because it binds to non-triple-helical collagen in angiogenic blood vessels. The investigation of drug-CREKA conjugate for the prevention and treatment of breast tumor in mice has been undergoing.				
<b>14. SUBJECT TERMS</b> No Subject Terms Provided.				<b>15. NUMBER OF PAGES</b> 9
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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## **DOD BCRP-01, Postdoctoral Trainee Award Progress Report Yr 03**

### **INTRODUCTION**

Toxic side effects limit the usefulness of many of the existing anti-cancer drugs. If it were possible to selectively target the drug into the tumor tissue, the efficacy of anti-tumor therapies could be enhanced while simultaneously decreasing the side effects. The homing peptide technology provides a new targeting strategy that aims at physically concentrating therapeutic agents in tumor tissue by making use of the unique features of tumor vasculature (Ruoslahti, E., *Drug Discovery Today* 7: 1138-1142, 2002).

In vivo screening of phage displayed peptides library has proved to yield peptides homing specifically to the vasculature of tumor or any given organ (Ruoslahti, E., *Nat. Rev. Cancer* 2: 83-90, 2002). The homing peptides can be used in targeted delivery of therapeutic agents as drug-peptide conjugates. In addition, identification of receptors for homing peptides will provide information for developing the improved versions of homing peptides and their drug conjugates.

In this project, peptides that specifically bind to the vasculature of breast carcinoma in MMTV PyMT mice or normal breast tissue were isolated by in vivo screening of phage displayed peptide libraries. The work has focused an identification of putative receptors for peptides and characterization of peptide/receptor biochemical interactions. The peptide identified in this work may be useful for designing new therapies that specifically target breast cancer.

### **BODY**

The approved tasks for this project are:

**Task 1:** Identification of the receptors for the peptides specifically binding to the mouse breast vasculature.

**Task 2:** Characterization of the biochemical interaction of peptide ligands and the putative receptors.

**Task 3:** Prevention- and intervention-studies with doxorubicin/targeting peptide conjugates in MMTV PyMT transgenic mice.

The substantial progress with each of the Tasks has been made with the first year of grant support of this fellowship:

*Task1.* The in vivo screening of phage-displayed peptides library has been performed with breast carcinoma in MMTV PyMT mice. Several peptides emerged from this screening. The most interesting peptide is a pentapeptide with the sequence CREKA. The CREKA-displaying phage homed to breast cancer tissue about 130 times more efficiently than non-recombinant T7 phage, but did not home to other tissues in MMTV PyMT mice (Fig. 1A). It also homed to MDA-MB-435 human breast cancer cell xenografts grown in the mammary fat pad of nude mice with a 20-fold specificity over non-recombinant phage (Fig. 1B). The breast tumor homing of the CREKA phage

was specific by the criterion of ligand inhibition, because co-injecting synthetic free peptide (5 mg in 500  $\mu$ l of PBS) with the phage inhibited phage recovery from breast cancer tissue (Fig.1B).

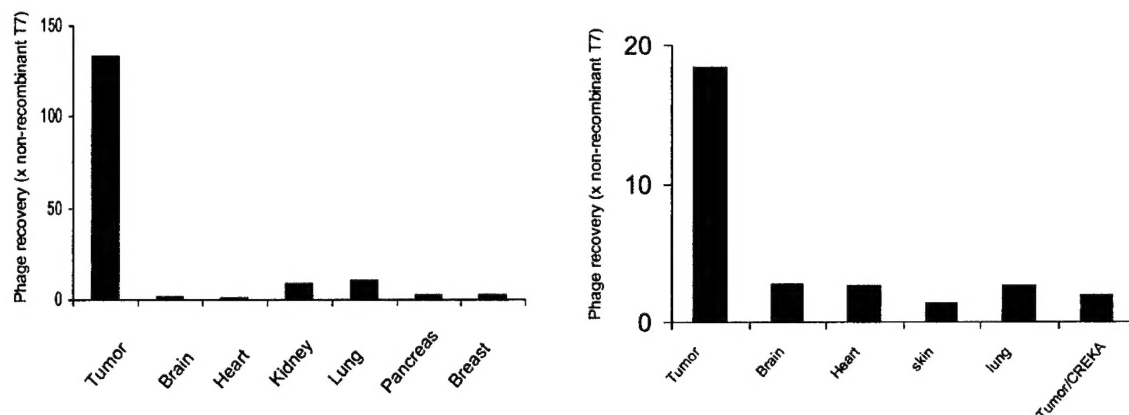


Figure 1. Specificity of the CREKA phage homing to tumors in MMTV PyMT mice (left) and mice bearing MDA-MB-435 xenografts (right). Phages ( $10^9$  pfu) were intravenously injected into mice, and were recovered from the tumors and control tissues. The number of pfu is shown. In some experiment, 5 mg of soluble CREKA peptide (Tumor/CREKA) was included in the injection. One representative experiment of four is shown.

Immunohistochemical staining revealed CREKA phage homing to the MMTV PyMT tumors (Fig. 2A) and in MDA-MB-435 xenografts (Fig. 2B) 15 min after intravenous injection. Non-recombinant T7 phage injected similarly could not be detected in the tumors (Fig. 2C); control staining with normal IgG was negative (Fig. 2D); and no CREKA phage was detected in control organs such as the brain (Fig. 2E), kidney (Fig. 2F), and heart (Fig. 2G). The liver stained positive for the CREKA (Fig. 2H) and for control phage (not shown), indicating non-specific uptake of the phage by the reticuloendothelial system, as has been noted before (Pasqualini, R. etc., *Cancer Res.* 60: 722-727, 2000).

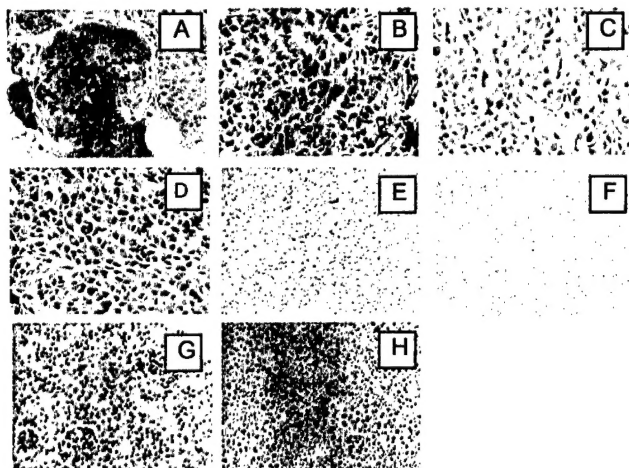
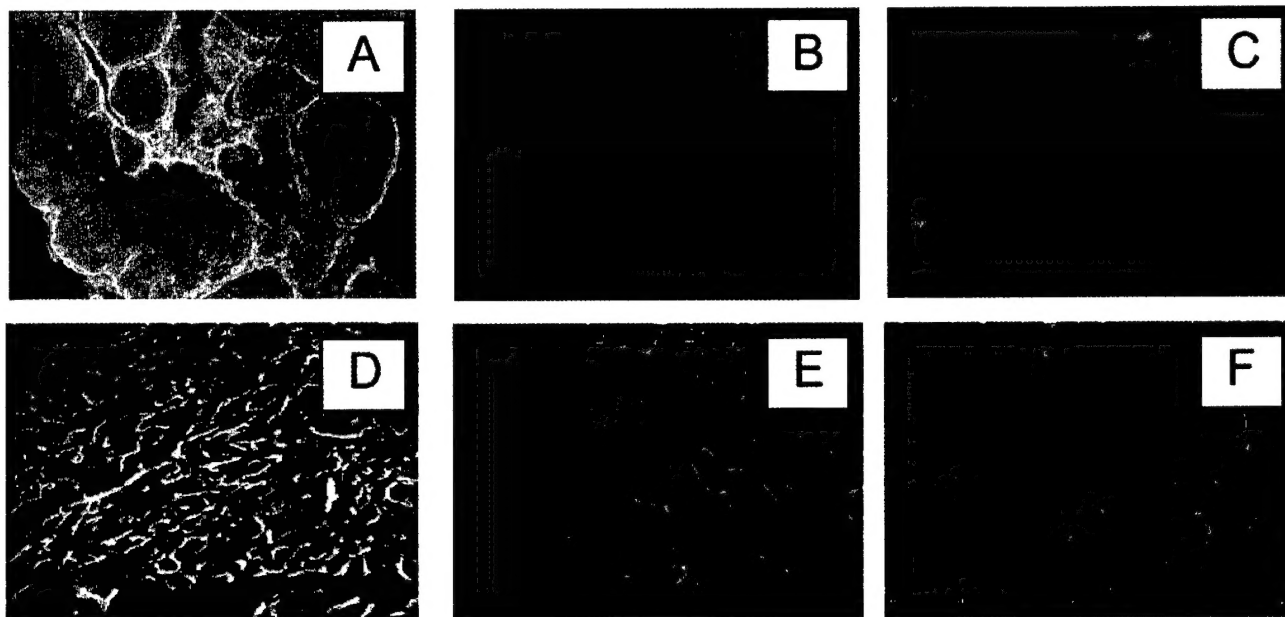


Figure 2. Localization of CREKA-displaying phage in mouse breast tumors. CREKA-phage ( $1 \times 10^9$  pfu) were injected into the tail vein of tumor-bearing mice. After 15 min of circulation, tumors and control organs were dissected, fixed, and stained with anti-T7 phage antibodies. CREKA phage was detected in MMTV PyMT mammary carcinomas (A) and in MDA-MB-435 xenograft tumors (B). Non-recombinant T7 phage could not be detected with antibodies in these tumors (C). Normal rabbit serum did not give any staining (D). CREKA phage could not be detected in non-tumor tissues: brain (E), heart (F), or kidney (G). T7-phage were detected in the liver, reflecting uptake of phage by the reticuloendothelial system (H).

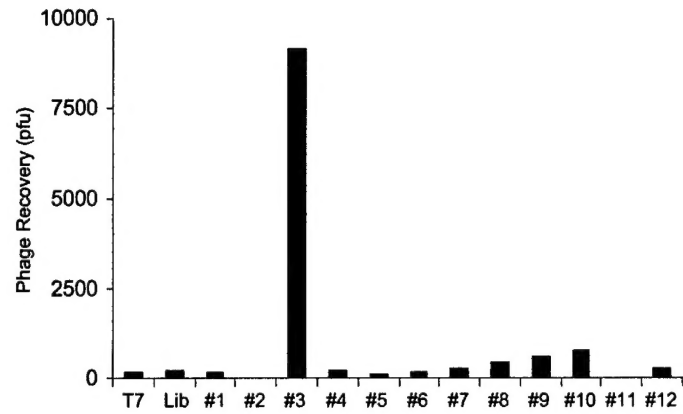
Furthermore, CREKA peptide labeled with fluorescein isothiocyanate (FITC) or rhodamine was detected in MMTV PyMT tumors 15 min after intravenous injection (Fig. 3A), but not in normal tissues such as the brain (Fig. 3B) or liver (Fig. 3C). The peptide was detected primarily in the tumor periphery 15 min after the injection, but spread into the entire tumor after 2 hr, localizing outside the blood vessels (Fig. 3D). FITC-labeled and rhodamine-labeled CREKA distributed in a similar pattern to different organs after in vivo circulation (data not shown). Peptide distribution in tumor tissue of a mouse co-injected with FITC-tomato lectin is shown in Figure 3E. Rhodamine-CREKA did not accumulate in control organs such as the heart (Fig. 3F). These data demonstrated that CREKA peptide homes to breast tumor.



**Fig.3. Fluorescein-labeled CREKA peptide homes to tumors, but not to normal tissues.** A-F, Localization of FITC-labeled and rhodamine-labeled CREKA peptide in MMTV -PyMT tumors. Fluorescein-labeled CREKA peptide (FITC-CREKA; 100  $\mu$ g in 100  $\mu$ l PBS) was injected into the tail vein of MMTV PyMT mice. After 15 min (A-C) or 2 hrs (D) of circulation, the mice were perfused through the heart. Organs were dissected, fixed, and cryo-sections were examined by fluorescence microscopy. In (D), nuclei were counter-stained with DAPI and tumor vasculature was visualized with intravenous injection of FITC-tomato lectin. Tumor vasculature visualized with intravenous injection of FITC-tomato-lectin (E). Heart from a MMTV PyMT mouse injected with rhodamine-CREKA and FITC tomato-lectin counter-stained with DAPI (F).

To identify the receptor for the CREKA peptide in breast tumor vasculature, a mouse breast cancer cDNA library for binding of the expressed proteins was screened to immobilized CREKA peptide. Among the individual phage clones obtained, one clone avidly bound to the peptide-coated surface (Fig. 4A), but not to an uncoated surface treated with the blocking buffer only. Sequence analysis showed that this clone encodes a 138-amino acid protein fragment related to the collagen IV alpha 2 chain (Fig. 4B). The presence of Gly-X-Y repeats showed that the fragment is derived from the triple helical portion of the collagen. These results suggest CREKA bind to type IV collagen in tumor blood vessels and tumor matrix. Others have shown that the mRNAs for several collagen chains, including type IV, are greatly (more than 30-fold) elevated in tumor endothelial cells compared to endothelial cells from adjacent normal tissue (St. Croix *et al. Science* 289, 1197-1202, 2000). This quantitative difference alone may explain the selective homing of CREKA-phage and labeled peptide to tumors. It is also possible that tumor collagens are poorly assembled in tumor tissue and expose more binding sites for the peptide. Further characterization of the CREKA peptide is underway.

A



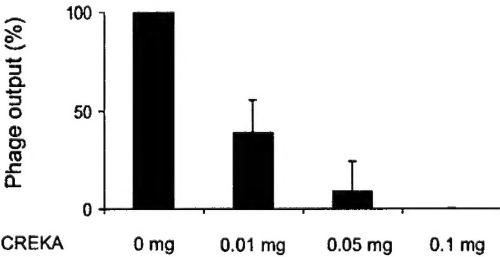
**Fig. 4. Specific binding of CREKA phage to a type IV collagen fragment.** (A) Biotin-labeled CREKA was immobilized on Streptavidin-coated ELISA plates. A cDNA library from mouse breast carcinoma in T7 phage ( $1 \times 10^8$  pfu) was applied to the CREKA-coated surface. The wells were washed with PBS, and specifically bound phage was eluted with an excess of soluble CREKA peptide. The eluted clones were amplified and individually tested with non-recombinant T7 phage (T7) and the original cDNA library (Lib) for binding to CREKA coated wells. Clone #3 phage avidly bound to CREKA. (B) The cDNA from the CREKA-binding phage encodes a 138-amino acid fragment with a sequence identical to the collagen IV alpha 2chain. The numbers that bracket the alignment denote the amino acid residues in collagen IV alpha 2 chain (NCBI accession number P08122) that align with clone #3.

B

Clone #3	GERGEQGPPGPSVYSPHPSLAKGARGDPGFQGAHGEPGSRGEPGEPGTAG
Collagen alpha 2 (IV)	336 GERGEQGPPGPSVYSPHPSLAKGARGDPGFQGAHGEPGSRGEPGEPGTAG 385
Clone #3	PPGPSVGDEDSMRGLPGEMGPKGFSGEPGSPARYLGPPGADGRPGPQGVP
Collagen alpha 2 (IV)	386 PPGPSVGDEDSMRGLPGEMGPKGFSGEPGSPARYLGPPGADGRPGPQGVP 435
Clone #3	GPAGPPGPDGFLFGLKGSEGRVGYGPSGFPGTRGQ - AW
Collagen alpha 2 (IV)	436 GPAGPPGPDGFLFGLKGSEGRVGYGPSGFPGTRGQKGW 474

*Task2.* The next study is to characterize the specificity of the interaction between CREKA and the collagen IV fragment. The interaction between CREKA-phage and Collagen was specific, as it could be inhibited by an excess of cognate CREKA peptide in a dose-dependent manner (Figure 5A). Rabbit anti-mouse collagen IV antiserum, but not control serum, blocked the interaction between the phage-displayed collagen fragment and immobilized CREKA. Fibronectin, which binds to various collagens in their non-triple helical form did not significantly affect the binding of the CREKA phage (Fig. 5B).

A



B

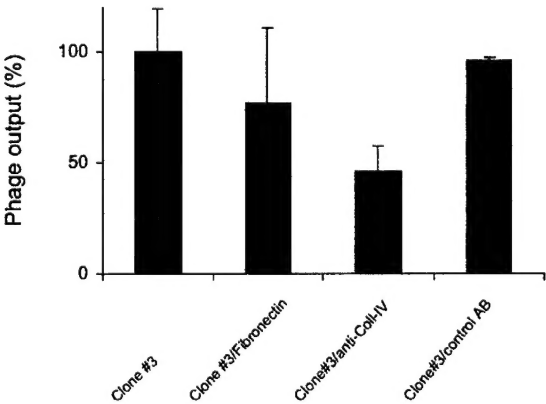




Figure 5. Specificity of binding to CREKA. (A) Inhibition of the CREKA collagen interaction by soluble CREKA peptide. Phage displaying the clone #3 collagen IV fragment ( $1 \times 10^7$  pfu) were applied to CREKA-coated 96-well ELISA plates and incubated for 1 hr in the presence of the indicated concentrations of soluble CREKA peptide determined. Phage bound to wells in the absence of peptide was set at 100% (means; n=3; bars, SE). (B) Binding of clone #3 to CREKA-coated surface in the presence of fibronectin (100  $\mu$ g/ml), anti-collagen IV antibodies 1:30, or normal serum (means; n=3; bars, SE).

Co-injecting gelatin (denatured collagen I) with the CREKA phage completely blocked in vivo tumor homing of the phage (not shown) suggesting that CREKA may preferentially bind to non-helical collagen in tissue.

**Task 3.** An independent study was carried out at Anticancer Inc. in San Diego, California compared the tumor homing of fluorescent-labeled CREKA and two other peptides Lyp-1 (Laakkonen, P. et al. *Nature Med* 8: 743-751, 2002) and F3 (Porkka, K. et al. *PNAS* 99: 7444-7449, 2002) from our laboratory. CREKA was found to be the most efficient and selective homing to MDA-MB-435 breast cancer xenografts.

We have also collaborated with Dr. Huber (Max Planck Institute for Biochemistry, Germany) in preparing a novel type of peptide-targeted conjugate. Dr. Huber's group incorporates cytotoxic amino acid to proteins that become toxic when the protein is degraded and the individual amino acid released. They have prepared CREKA-toxic GFP recombinant proteins for us which we are in the progress of testing for in vivo antitumor activity.

## KEY RESEARCH ACCOMPLISHMENTS

- Characterized a pentapeptide, CREKA, which specifically homes to breast tumor vasculature
- Identified the type IV collagen as the receptor for CREKA peptide that homes to breast cancer vasculature
- Shown that a fluorescein-labeled peptide of CREKA that recognizes vasculature in breast cancer xenografts specifically accumulates in the tumor with an superior efficacy after an intravenous injection.

## CONCLUSIONS

The significant progress has been made toward completing each of the Tasks in the application. A receptor for a peptide that homes to breast tumor vasculature is identified. A new blood vessel homing peptide show remarkably efficient and specific accumulation in breast cancer xenografts, boding well for future drug targeting experiments.

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